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Methionine is a Key Regulator in the Onset of Atopic Dermatitis in NC/Nga Mice

Takayuki Koga,*[a] Fuka Hirayama,[a] Tomomitsu Satoh,[b] Yuji Ishii,[c] Nobuhiro Kashige,[b] Makoto Hiromura,[a] Fumio Soeda,[a] and Akihisa Toda[a]

[a]*Daiichi University of Pharmacy, 22-1, Tamagawa-cho, Minami-ku, Fukuoka 815-8511, Japan;* [b]*Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1, Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan;* [c]*Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*
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Atopic dermatitis (AD) is a skin disorder that presents with itching and scratching and frequently progresses to a chronic state. AD often develops in patients with an individual or family history of allergic diseases. In addition, AD may develop in patients exposed to environmental stimuli such as air pollutants or dust. However, there can be differences in the magnitude of symptoms between patients even with the same genetic background or exposure to similar environmental conditions. NC/Nga mice have been used as a model for AD. They show AD-like symptoms in an age-dependent manner, even in the absence of AD-inducing agents. In addition, similar to humans, the magnitude of AD symptoms in this model varies between individual mice. However, the mechanisms underlying these differences are unclear. In addition, little is known about the relationship between AD skin symptoms and other organs and tissues. Here, we performed a metabolome analysis on sera from NC/Nga mice to identify factors potentially related to the severity of AD symptoms. The analysis showed a correlation between reduced serum methionine levels and increased severity of AD. In addition, treatment with excess methionine before the onset of AD symptoms suppressed the development of AD. In contrast, administration of methionine after the onset of AD symptoms did not. Importantly, cysteine and taurine, irreversible metabolites of methionine, did not suppress AD development. These results show that methionine, but not its metabolites, is a key factor in the onset, rather than the development of AD.

Key words atopic dermatitis, methionine, NC/Nga, metabolomics

INTRODUCTION

Atopic dermatitis (AD), also referred to as eczema, is a major skin disorder characterized by itching and has an exacerbation-remission cycle.[1)] It is estimated that 15-20% of children and 1-3% of adults have AD.[1,2)] There are numerous mechanisms involved in the development of AD, including genetics, environmental factors, and immune system dysfunction.[3,4)] Patients often have a genetic background that lends itself to allergic diseases, such as asthma, rhinitis, or atopic dermatitis itself.[2,5-7)] In addition, numerous genetic factors associated with the production of IgE in response to allergens, also contribute to the development of AD.[8,9)] The onset of AD is also related to exposure to environmental stimuli, such as air pollutants, house dust, and mites.[10-18)] Some studies have shown that the elimination of environmental stimuli or an avoidance of skin contact with those stimuli can lead to a reduction in the severity of AD symptoms, but others have reported that elimination is not sufficient to reduce symptoms.[19-27)] Because many factors participate in the onset and/or development of AD, it is difficult to understand the entirety of the disease. This is why appropriate treatments based on the mechanism of AD have yet to be developed, and the current treatments for AD only inhibit the disease symptoms without curing the disease itself.[28)] Currently, there are three therapeutic strategies for the treatment of AD in Japan: topical care to reduce physiological abnormalities of the skin, the elimination of allergens, and drug treatment.[29)] A combination of strategies is often used, based on the condition of the patient. The first approach, topical care, is aimed to repair the reduced skin barrier function caused by AD.[30,31)] A moisturizer is often used to help prevent a relapse in AD symptoms and/or to suppress itching.[32,33)] The second approach, elimination of allergens, is to avoid AD antigens in food and/or the living environment. However, the effects of this are still controversial, because some reports show that elimination of food antigens is not effective at reducing AD symptoms.[23)] In contrast, the elimination of antigens within the environment such as dust and mites, does appear to reduce AD symptoms in some cases.[19,20)] The third approach, drug treatment, with steroids or calcineurin inhibitors for example, is often used as topical medicines.[34-39)] Other drugs, including antihistamines, are used as internal medicines.[40)] These three strategies, especially drug treatments, work well to suppress AD symptoms, but they do not cure AD completely. Therefore, more research is needed to establish a clear strategy aimed towards a complete recovery from AD. Thus, the mechanisms underlying the etiology of AD including its onset and/or development need to be defined,

*To whom correspondence should be addressed. e-mail: ta-koga@daiichi-cps.ac.jp

in order to develop these new therapeutic strategies.

The magnitude of AD symptoms varies widely between patients, even those with similar genetic backgrounds such as family members and those living in the same environment.[41,42] However, the mechanism for why these individual differences in AD symptoms occur remains unclear. NC/Nga mice have been developed as an AD model and have been widely used for decades.[43,44] This mouse model shows pathological and behavioral features similar to human AD, including scratching/itching, erythema/hemorrhage, edema, crustiness/dryness, and elevated levels of total serum IgE, starting at approximately 8 weeks of age.[43,45] In some studies, AD-inducing agents or chemicals, such as mite antigens and halogenated polynitrobenzene, are used to induce clinical AD-like skin lesions in NC/Nga mice.[46-48] However, these mice can show AD-like symptoms even in the absence of AD-inducing agents. Under these conditions, some mice show severe AD lesions comparable to those seen in humans, while other mice show no symptoms even when living with mice with severe AD. Thus, the magnitude of AD symptoms in this mouse model varies between individual mice, similar to humans. However, the reasons why NC/Nga mice show varied AD symptoms between each mouse, just like humans, are not understood. It is likely that differences in skin symptoms also affect other organs/tissues, and that other organs and tissue also affect differences in skin symptoms. Blood is well known to connect organs and tissues to one another, including the skin. If differences in skin symptoms affect other organs/tissues, it is logical to conclude that blood/serum would also be affected. This led us to hypothesize that there would be differences in the serum of individual mice that correlate to the severity of AD symptoms. In this study, we used a metabolomic analysis to try to identify the mechanism underlying the differences in AD severity in NC/Nga mice. We also investigated differences in serum components that correlated with the severity of AD symptoms. Finally, we investigated the importance of the essential amino acid methionine and its metabolites on the onset and development of AD symptoms.

MATERIALS AND METHODS

Materials Methionine, cysteine, and taurine were purchased from Nacalai Tesque (Kyoto, Japan). The other reagents were of the highest grade commercially available.

Animals and Treatments All experiments were approved by the Institutional Animal Care and Experiment Committee of the Daiichi University of Pharmacy (approval No: 14003 and 17006). Male NC/Nga mice (5 or 9 weeks old) were purchased from Japan SLC Co. Ltd. (Shizuoka, Japan). All mice were used after 1 week of acclimation. In some experiments, the mice were housed for 6 weeks with five mice/cage. Following this period, mice were separated so that there was one mouse/cage to measure food consumption for 24 h. The next day, the magnitude of AD severity was assessed using a clinical skin score, after which sera were collected for additional experiments. The clinical skin score for AD-like lesions were defined as previously reported with some modifications.[43,49] Briefly, the four typical signs and symptoms of AD, scratching/itching, erythema/hemorrhage, edema and crustiness/dryness, were individually scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). Then, these four scores were summed to provide the severity score.

In a separate experiment, the effects of methionine, cysteine, or taurine on the onset or development of AD were examined. In the methionine experiment, 6 or 10-week-old male NC/Nga mice were housed until 16 weeks of age and the skin severity score was determined once a week as described above. Methionine was administered in the drinking water at a concentration of 30 mg/L during the housing period, from 6 or 10 weeks of age to 16 weeks of age. The concentration was decided based on the recommended daily intake of methionine by the World Health Organization[50] and the volume of water consumption in this mouse model. At the end of the treatment period, sera and the scratched regions of skin around the flank of the mice were collected. The skin regions were fixed with 10% neutral buffered formalin for 24 h at around 20-25°C. All collected sera were stored at -80°C until use. Experiments with cysteine and taurine were performed as described above for methionine at a concentration of 30 mg/L each.

Metabolomics Analysis Changes in the metabolomic profile were analyzed by procedures described previously with some modifications.[51] Briefly, serum was collected from each mouse. After spiking with 150 ng glycylnorleucine as an internal standard, the serum components were extracted with an equal volume of methanol, and dried in a vacuum concentrator. The residue was reconstituted in an acetonitrile/H_2O mixture (acetonitrile: H2O = 1:1), and an aliquot was subjected to high-performance liquid chromatography coupled with a time-of-flight mass spectrometry (HPLC-TOF-MS) analysis. The operating conditions of HPLC were as follows: column, BEH C18 column (1.7 μm particle size, 2.1 × 100 mm, Waters, Manchester U.K.); column temperature, 40°C; sample room temperature, 10°C; mobile phase, changing acetonitrile ratio (%, v/v) in 10 mM ammonium formate as follows: 2% (0-8 min), 2 to 98% (8-16 min), 98% (16-18 min) and then stepwise returning to 2% and maintaining this for 3 min; flow rate, 0.2 mL/min. The operating conditions of TOF-MS were as follows: ionization, electro-spray ionization with capillary voltage at 3.5 kV in positive ion mode; cone and multiplier voltages, 50 and 550 V, respectively; source temperature, 120°C; desolvation temperature, 350°C. The spectra were recorded by detecting ions ranging from m/z 50 to 1,000. Metabolome profiles were compared with each other using principal component analysis (PCA), as described previously.[51] The information including the retention times and intensity of ions obtained by HPLC-TOF-MS was normalized by the internal standard, and converted to a data matrix capable of being compared with data from other samples using MarkerLynx™XS software (Waters). The data matrices from different mice were subjected to PCA, using the same software (Fig. 3A). To compare the metabolome profile between each group, these data were analyzed using an orthogonal partial least-squares to latent structures-discriminant analysis (OPLS-DA) method using a combination of MarkerLynx™XS and SIMCA-P software (MKS-Umetrics, Malmo, Sweden). From the OPLS-DA score obtained, fragment ions that contributed to the severity of AD-dependent alteration were plotted (S-plot, Fig. 3B). On this basis, serum components that contribute to the severity of AD-dependent changes were identified by selecting compounds making a contribution to the correlation co-efficient which was either more than +0.8 or less than -0.8. The compounds detected above were identified by referring to the retention time and mass information available in online databases, such as the Human Metabolome Database (http://www.

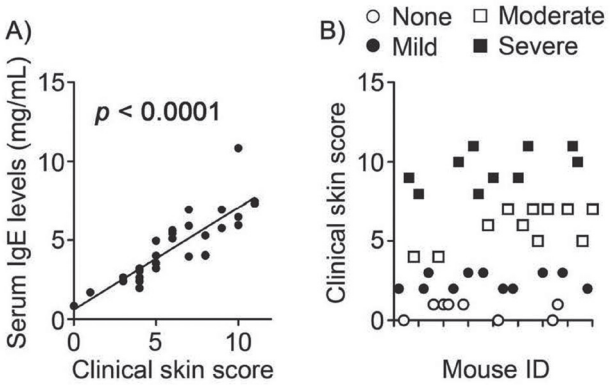

Fig. 1. Diverse Degrees of the Severity of AD in Individual NC/Nga Mice in the Absence of AD-Inducing Agents

(A) Correlation analysis of the clinical skin score with serum IgE levels in 16-week-old mice. (B) Clinical skin score plots for each mouse at 16 weeks of age. Mice were then dived into four groups based on the severity of AD seen: None (open circles), Mild (closed circles), Moderate (open squares), and Severe (closed squares). Each dot indicates a different animal (N = 8-11/group).

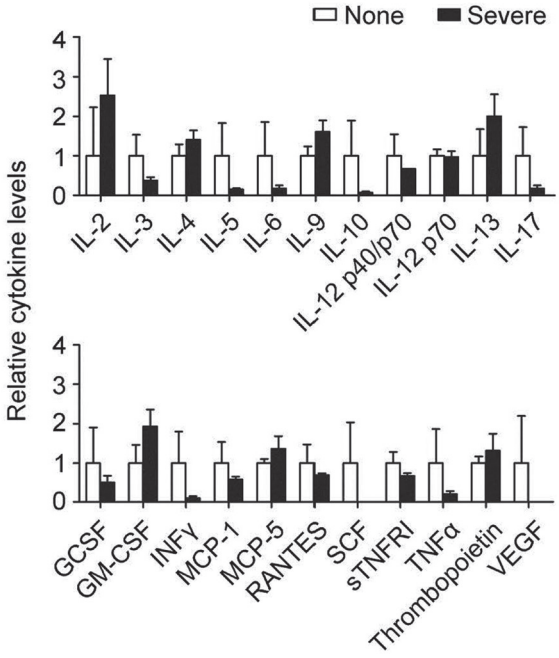

Fig. 2. Severity of AD Disturbs Serum Cytokine Levels

Cytokine arrays showing the AD-induced disturbance of cytokine levels, indicating a disturbance in the Th1/Th2 balance. Each bar represents the mean ± SD (N = 3), normalized with the values of the "None" group. Abbreviations; IL, interleukin; GCSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; INF-γ, interferon-gamma; MCP, monocyte chemoattractant protein; RANTES, regulated on activation, normal T cell expressed and secreted; SCF, stem cell factor; sTNFRI, soluble tumor necrosis factor receptor I; TNFα, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

hmdb.ca/) and the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/). As for measure of serum methionine levels, serum samples were extracted with the same procedure, and were measured with the same conditions of LC and TOF-MS, as mentioned above. The methionine levels were calculated based on the standard curve obtained from the serial dilution of the standard solution of methionine.

Histological Evaluation A section of skin was removed from the left flank, fixed in a neutral 10% formaldehyde buffer solution (Nacalai Tesque, Inc., Kyoto, Japan), embedded in paraffin, and sectioned to 5 μm thickness. The sections were stained with Mayer's Hematoxylin Solution and Eosin Y solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Statistical Analysis Statistical differences were assessed using either Student's *t*-test to compare two groups, or a two-way ANOVA with a Bonferroni *post hoc* test to compare clinical skin scores. Statistical correlations were determined using Pearson's correlation coefficient.

Other Methods Serum cytokine levels were measured using a mouse cytokine antibody array containing 22 target proteins (ab133993; Abcam, Cambridge, UK) following the manufacturer's protocol. The serum levels of IgE and histamine were determined by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (IgE, FUJIFILM Wako Shibayagi Corp., Tokyo, Japan; histamine, Enzo Life Sciences Inc., Farmingdale, NY).

RESULTS

Effects of AD Severity on Serum Metabolic Profiles Prior to determining which serum components or factors correlate with the severity of AD symptoms, we confirmed that NC/Nga mice housed until they were 16 weeks old without treatment were an appropriate model for this study. First, we confirmed that the clinical skin score we used was adequate to evaluate the severity of AD. We used serum IgE levels to evaluate the degree of AD development, because Matsuda *et al*. have reported that AD development in NC/Nga mice is associated with elevated IgE levels in the blood.[43] In the present study, the serum levels of IgE were significantly correlated to the AD clinical skin score with a p value of less than 0.0001 (Fig. 1A). Therefore, based on serum IgE levels, the clinical skin score we used correctly reflected the degree of AD severity. Next, we evaluated the development of AD symptoms using the clinical skin score. We housed 40 mice (5 mice/cage) from 10 weeks to 16 weeks of age and evaluated their AD symptoms using the skin score. Under these conditions, the severity of AD symptoms was variable. Although several mice had significant AD symptoms on their skin, others did not. The highest skin severity score was eleven, and the lowest was zero (Fig. 1B). We then divided the mice into four groups based on their skin score for an additional analysis: 0 to 1 = None; 2 to 3 = Mild; 4 to 7 = Moderate; > 8 = Severe (Fig. 1B). It has been reported that in NC/Nga mice, the development of AD results from a disturbance in the balance of helper T cell (Th) Th1/Th2 balance.[52] We confirmed that the Th1/Th2 balance in the "Severe" group of AD-mice was disturbed compared with the mice in the "None" group (Fig. 2). For example, interferon-gamma (INF-γ), a Th1 activation cytokine, was decreased by AD development, while interleukin-13 (IL-13), a Th2 activation cytokine, increased. These results show that NC/Nga mice housed until 16 weeks of age without treatment were an appropriate model to determine the factors or components involved in the differences in AD severity.

We next carried out a metabolomic analysis using HPLC-TOF-MS to identify differences between mice that did or did not develop AD symptoms. The mice were divided into four groups (Fig. 1B) and serum components were analyzed in positive ion mode. The principal component analysis of the HPLC-TOF-MS data showed that development of AD altered

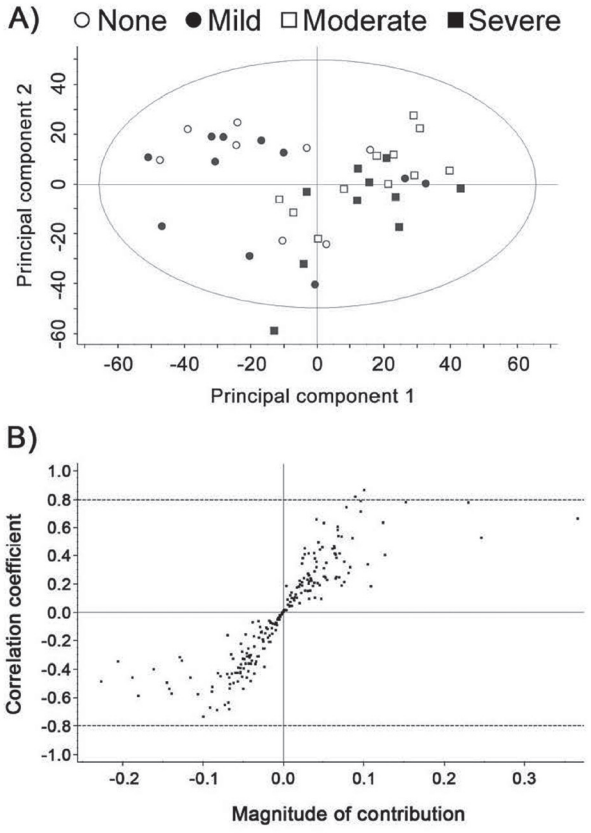

Fig. 3. The Severity of AD Only Slightly Alters the Serum Metabolome Profiles by Principal Component Analysis (PCA)

(A) PCA plots of the serum metabolomes. Each dot is a different animal (N = 8-11/group). (B) Fragment ions in the HPLC-TOF-MS analysis showing a difference when comparing the "None" and "Severe" groups. Each dot shows a single ion with a particular mass (*m/z*). The criteria for selecting ions that were significantly changed in the "Severe" group compared with the "None" group was set as being either more than 0.8 or less than -0.8 of the correlation coefficient.

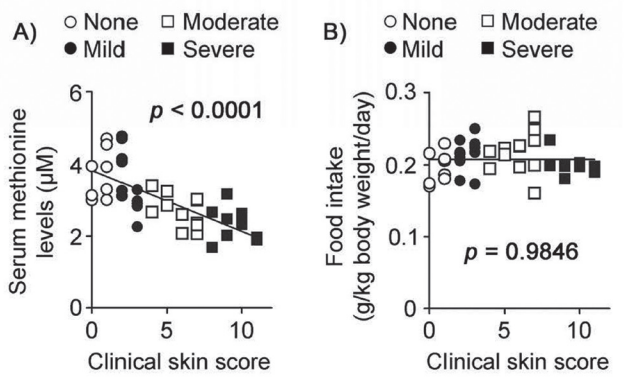

Fig. 4. Serum Methionine Levels are Negatively Correlated with the Severity of AD Symptoms

(A) A correlation analysis of the clinical skin score with serum methionine levels, showing that these two parameters are significantly correlated. (B) Correlation analysis of the clinical skin score with daily food intake, showing that these two parameters are not significantly correlated.

the metabolomic profile in serum; however, the effects were limited (Fig. 3A). Supporting this, the "S-plot" showed that only a few ions were detectable at levels that were significantly changed by AD development (Fig. 3B). We observed a decreased ion at m/z 150.0586 and identified it as methionine by referring to its fragment pattern and the retention time of a methionine standard. Quantification of serum methionine levels based on calibration with standards showed that the serum levels of methionine were significantly and negatively correlated with the degree of AD symptoms (Fig. 4A, $p < 0.0001$). Methionine is an essential amino acid that is acquired only from food. Importantly, the severity of AD did not affect food intake by the animals in this study (Fig. 4B, $p = 0.9846$).

Effects of Methionine on AD Onset/Development To determine how methionine affects the symptoms of AD, we examined the effects of excess methionine administration on AD severity. Oral administration of methionine led to a temporary elevation in serum methionine levels, but these rapidly decreased due to distribution and metabolism in organs.[53] Therefore, in order to maintain a condition with an excess intake of methionine, we administered it through the drinking water, rather than by transient oral administration. The administration was performed from 6- ("Pre" group) or 10 weeks of age ("Post" group) up to 16 weeks of age, the age by which the symptoms of AD are well developed (Fig. 5A). Methionine administered from 6 weeks of age, i.e. before the onset of AD, significantly suppressed the development of AD symptoms from 12 weeks to the end of the observation period (Fig. 5B). However, administration of methionine from 10 weeks of age, i.e. after the onset of AD, did not suppress the development of AD symptoms (Fig. 5C). In NC/Nga mice, development of AD causes hyperplasia of the epidermis with a severity related to the degree of AD symptoms.[43] In the "Pre" group, administration of methionine before the onset of AD symptoms slightly but significantly suppressed epidermal swelling (Fig. 6). In the "Post" group, this suppression of epidermal swelling was not observed (Fig. 6). These results suggest that suppression of AD via methionine depends on the time of administration as shown in Fig. 5. As discussed previously, development of AD is followed by an elevation in serum IgE levels.[43] The "Pre" group showed a suppression of this elevation in of serum IgE levels, whereas the "Post" group did not (Fig. 7A). In contrast, methionine did not reduce serum histamine levels, regardless of the administration procedure used (Fig. 7B). Our results indicate that methionine suppresses the development of AD in an administration time-dependent manner.

Effect of Methionine Metabolites on the Development of AD To address the mechanism by which methionine suppresses AD, we examined the effects of cysteine and taurine, both of which are irreversible metabolites of methionine. If methionine exerts its suppressive effect on the development of AD development via the metabolism of methionine, it would be expected that cysteine and taurine would also suppress the development of AD. As shown in Fig. 8, neither cysteine nor taurine suppressed the development of AD, even though the administration procedure was the same as that used for methionine (Fig. 5).

DISCUSSION

AD is an inflammatory skin disorder characterized by exacerbation-remission cycles and often progresses to a chronic state. The mechanisms underlying the development of AD development are complicated, because many factors, such as immune system dysfunction, environmental exposure,

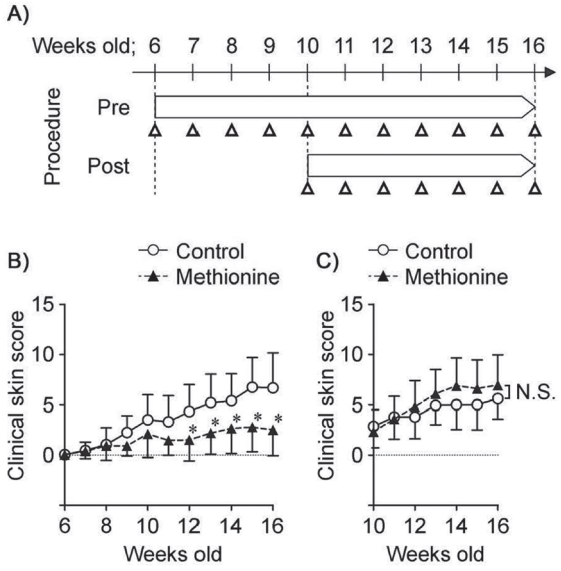

Fig. 5. Methionine Suppresses Increased Clinical Skin Scores in an Administration Time-Dependent Manner

(A) Experimental procedure used for methionine administration, showing administration from 6 to 16 weeks of age ("Pre" group) compared with administration from 10 to 16 weeks of age ("Post" group). Open triangles represent the clinical skin score assessment time points. The scores were assessed every week. (B) Temporal changes in clinical skin scores in the "Pre" group are shown in panel A, showing that methionine significantly suppresses the development of AD from at least from 12 weeks of age. (C) Temporal changes in clinical skin scores in the "Post group" are shown in panel A, showing that methionine does not significantly suppress the development of AD over the procedure period. Each plot represents the mean ± SD (N = 15). * $p < 0.05$ compared with the control group. N.S., not significant.

and genetics are thought to be involved, however, the specific details remain unclear. Therefore, the current AD therapies do not aim for a complete recovery, but only suppress the symptoms. In other words, appropriate treatments based on the mechanisms involved in the development of AD have not been established because the mechanisms are still unclear. Therefore, clarifying the detailed mechanisms underlying the development of AD is necessary to develop more effective AD therapies. In this study, we used NC/Nga mice which had not been treated with any AD-inducing agents. Importantly, in this mouse model there is a spectrum of clinical severity scores for AD, even though all mice are maintained in the same environment. This is similar to the situation in human AD, where symptoms vary in each patient, even under the same environmental conditions or in patients from the same genetic background. Recently, AD was shown to be not only a skin disease but also that affects the function of other organs.[54,55] Conversely, other organs could also affect the magnitude of AD.[56] These findings led us to hypothesize that AD in the skin and in other organs can affect each other in terms of magnitude of function/dysfunction or symptoms. If this is true, there are likely mediating factors between the skin and other organs that reflect the severity of AD symptoms and organ function. The levels of these mediating factor(s) are presumed to correlate with the severity of AD. Thus, we hypothesized that these factor(s) might play a critical role in the individual differences seen in the severity of AD.

Metabolomic analyses can identify alterations in even small levels of compounds, and can be used to define mechanisms and diagnostic markers in numerous diseases However, there are only a few reported studies using this method in AD, and these have not focused on individual differences in the severity of AD.[57,58] The blood stream is the major carrier of mediators from organ to organ. For example, nutrients absorbed in the small intestine are distributed to the whole-body via the blood stream. Therefore, we hypothesized that the mediating factors described above might also be carried by the blood stream. Accordingly, in this study, we performed metabolomic analyses using sera to identify factors that correlated with the severity of AD symptoms. We found that the levels of methionine, an essential amino acid, decreased in a severity of AD dependent manner (Fig. 4A). However, our results about serum methionine levels in Fig. 4A were much lower than methionine levels already reported elsewhere,[59,60] including the None group. We are not sure about the reason for this dif-

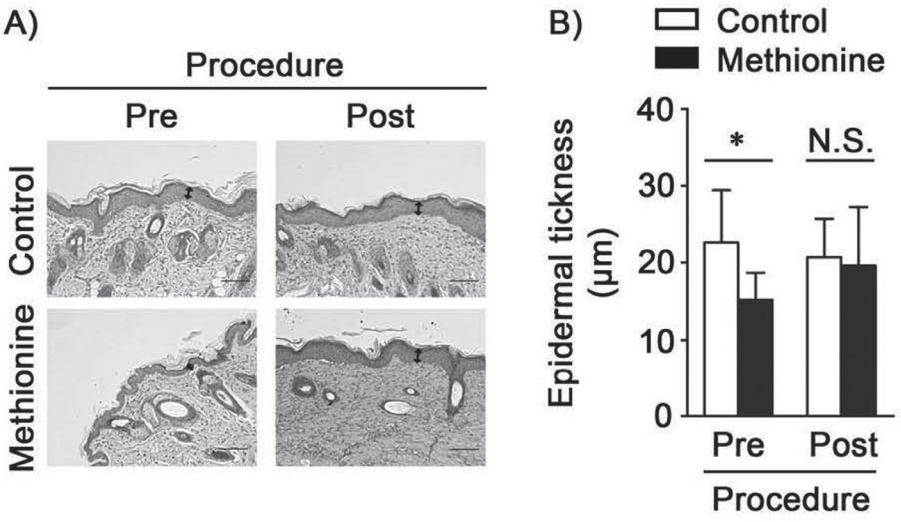

Fig. 6. Methionine Suppresses Epidermal Swelling in an Administration Time-Dependent Manner

(A) Skin sections of each mice following HE staining, showing that methionine suppresses the epidermal swelling caused by the development of AD in a procedure-dependent manner. The two-direction arrows indicate the epidermal thickness measured in panel B. (B) The thickness of epidermis measured on the skin section as shown in panel A. Each bar represents the mean ± SD (N = 15). * $p < 0.05$ compared with indicated pairs. N.S., not significant.

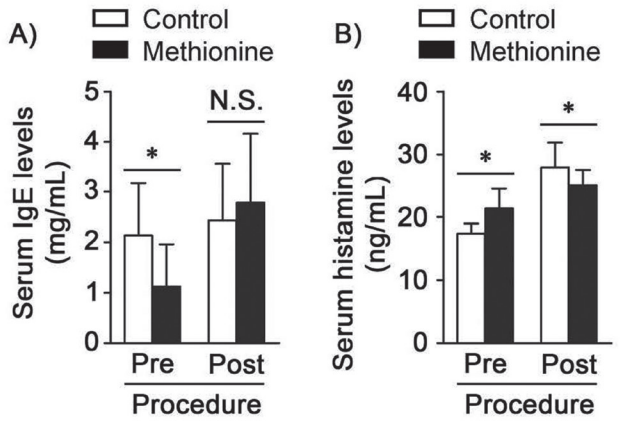

Fig. 7. Methionine Modulates Serum IgE and Histamine Levels in an Administration Time-Dependent Manner

(A) The serum levels of IgE as an indicator of AD severity, showing that methionine decreases serum IgE levels only in the "Pre" group as shown in Fig. 5A. (B) The serum levels of histamine, a mediator that also indicates the severity of AD, showing that methionine slightly but significantly modified serum histamine levels in a procedure-dependent manner. Methionine increased serum histamine levels in the "Pre" group, and decreased their levels in the "Post" group. Each bar represents the mean ± SD (N = 15). * $p < 0.05$ compared with indicated pairs. N.S., not significant.

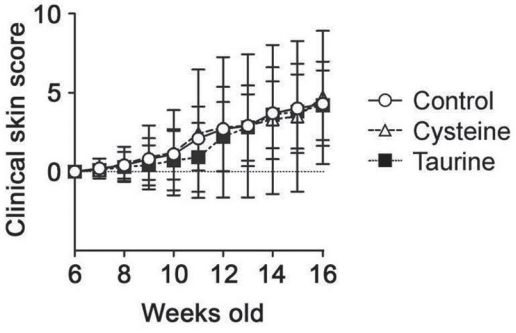

Fig. 8. Irreversible Metabolites of Methionine Do Not Suppress the Increase in Clinical Skin Score in AD

Temporal change in clinical skin scores in the "Pre" group shown in Fig. 5A, indicating that cysteine and taurine, both irreversible metabolites of methionine, do not suppress the development of AD, even though in the same procedure methionine does, as shown in Fig. 5B. Each plot represents the mean ± SD (N = 10).

ference, but at least the fact that methionine levels decreased with AD severity would remain the same. Normally, the levels of essential amino acids in the body are directly affected by food consumption, because animals cannot biosynthesize essential amino acids. However, in this study, the food intake for each mouse did not affect the severity of AD symptoms (Fig. 4B). This indicates that the decrease in serum methionine levels did not depend on absorption, but rather on other steps, such as distribution, metabolism, or excretion. Unfortunately, we could not identify which step was important for the decrease in methionine levels. However, since the methionine levels and the severity of AD were significantly correlated, the decrease in methionine levels appeared to be a mediator reflecting the severity of AD symptoms. If this hypothesis is true, inhibiting the decrease of methionine levels could relieve AD symptoms. Since it was likely that a transient administration of excess methionine, which would be achieved through an oral gavage, and their rapid distribution throughout the body would be unlikely to overcome the decrease of methionine in levels seen in AD,[53] we elected to administer excess methionine through the drinking water. Using this administration route, we found that methionine suppressed the development of AD symptoms but only in the "Pre" group, where administration was begun at 6 weeks of age, before the onset of AD symptoms. In the "Post" group, in which methionine administration began after the onset of AD (10 weeks of age), no suppression of AD symptoms was observed This result was supported by decreases in serum IgE levels in the "Pre" group, but not in the "Post" group. These results also indicate that methionine itself does not suppress IgE release directly, because methionine administered to the "Post" group did not show reduced IgE levels (Fig. 7A) Unfortunately, the mechanism by which methionine suppresses the development of AD remains unclear. Histamine is well known to be an inflammatory cytokine, and its release is enhanced by the IgE-antigen complex.[61] Histamine is also known to be an inducer of AD symptoms,[62] and is often used as an indicator to evaluate the efficacy of therapeutic treatments. However, in the "Pre" group, the serum levels of histamine were slightly increased, even though IgE levels were significantly decreased (Fig. 7A and B). These results suggest that methionine suppresses the development of AD independently of the well-known IgE-histamine pathway. Since methionine administered pre-onset suppressed AD symptoms, but post-onset it did not, this suggests that methionine suppresses AD symptoms at the AD onset step but not at the AD development steps.

In the body, methionine is converted to *S*-adenosylmethionine by methionine adenosyltransferase, followed by conversion to homocysteine by methyl transferase and *S*-adenosylhomocysteine hydrolase. Following this, homocysteine is converted to methionine again by betaine-homocysteine methyltransferase or methionine synthase.[63] These reactions are referred to as the methionine cycle. Homocysteine is also converted to cystathionine by cystathionine-β-synthase, which is then converted to cysteine by cystathionin-γ-lyase, and finally to taurine. The synthesis of cysteine and taurine are catalyzed in an irreversible manner, in contrast to the reversible reactions of the methionine cycle.[63] In this study, we also investigated whether these irreversible metabolites of methionine participate to suppress the symptoms of AD (Fig. 5). Neither cysteine nor taurine suppressed the development of AD, even though the administration route, dose, and period were the same as used for methionine (Fig. 8). This result indicates that the suppression of the development of AD by methionine is not due to its metabolites, but rather to methionine itself or perhaps the methionine cycle.

In conclusion, the present study demonstrates that methionine plays an important role in the severity of AD. The fact that methionine levels are negatively correlated with the clinical severity score for AD suggests that methionine could be a novel marker of AD severity. Since the severity of AD is defined by markers, including clinical score and serum IgE levels, this novel marker could support diagnosis more accurately. In addition, the fact that methionine administered

before the onset of AD symptoms but not after the onset of AD symptoms can suppress the development of AD symptoms suggests that methionine or its analogs could be used as a preventive medicine rather than a remedial medicine.

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Conflict of interest The authors declare no conflict of interest.

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